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Purification and In Vitro Phosphorylation of *Myxococcus xanthus* AsgA Protein

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The deduced amino acid sequence of the *Myxococcus xanthus* AsgA protein contains an N-terminal domain that is homologous to the receiver of response regulators and a C-terminal domain that is homologous to the transmitter of histidine protein kinases. We overexpressed affinity-tagged AsgA in *Escherichia coli*, purified the recombinant protein, and showed that AsgA has autokinase activity in vitro. The results of chemical-stability assays suggest that AsgA is phosphorylated on a histidine and provide no evidence for transfer of the phosphoryl group to the conserved aspartate of the receiver domain.

Fruiting-body development in *Myxococcus xanthus* requires communication between tens of thousands of cells. Accordingly, there is evidence for at least five intercellular signals that act sequentially over the course of development (1, 3, 6). The A-signal is required early in fruiting and has been identified as a mixture of amino acids and small peptides generated by extracellular proteolysis (9, 13). It is thought that the A-signal allows *M. xanthus* to determine whether the cell density is sufficiently high for fruiting-body formation (8).

At least three genes, *asgA*, *asgB*, and *asgC*, are required for the production of A-signal. Our laboratory has been focusing on the mechanism by which the *asg* genes are involved in A-signal production. This study concerns the product of the *asgA* gene. The deduced amino acid sequence of AsgA (14) is strikingly similar to those of a highly conserved group of signal transduction proteins of the so-called two-component regulatory systems (11, 12, 17), which regulate a wide variety of bacterial adaptive responses. The paradigm two-component system consists of a pair of enzymatic components: one is a sensor protein containing a highly conserved histidine protein kinase (HPK) domain, or "transmitter"; the other is a response regulator (RR) which contains a highly conserved domain, or "receiver."

AsgA belongs to a subclass of sensor proteins that possess not only a conserved histidine-containing transmitter domain but also a conserved aspartate-containing receiver domain (12, 14). However, AsgA exhibits features distinct from those of other members of this subclass. It lacks the membrane-spanning regions that are characteristic of many sensor proteins in the two-component family. Most interestingly, AsgA contains neither an input domain, which is characteristic of all other known sensors, nor an output domain, which is characteristic of most RR proteins. In addition, AsgA displays a unique domain organization, with its conserved RR domain located at the N terminus and the conserved HPK domain at the C terminus. We hypothesize that AsgA interacts with other, unidentified signaling proteins that provide input and/or output functions (for a discussion of this hypothesis, see reference 14).

We describe here the purification and in vitro phosphorylation of *M. xanthus* AsgA. In addition, we used chemical-stability assays to investigate the nature of the phosphorylated amino acid residue in AsgA-phosphate. Our results suggest that the phosphate is linked to a histidine.

Construction of the expression plasmid for AsgA protein. Plasmid pQE30 (QIAexpress system; Qiagen Inc.) is designed for high-level expression in *Escherichia coli* of proteins containing six consecutive histidine residues (6xHis affinity tag). A plasmid for overexpression of AsgA containing an N-terminal 6xHis tag was constructed as follows. Plasmid pQE30 was digested with *Bam*HI, treated with Klenow and deoxynucleoside triphosphates to fill in the ends, and then digested with *Kpn*I. The linear form was purified from an agarose gel. Plasmid pLP11 (14), which contains the *asgA* gene, was digested with *Bsp*HI, treated with Klenow to fill in the ends, and then digested with *Kpn*I. A 1.7-kb fragment containing the entire coding region of *asgA* was isolated from an agarose gel. This fragment was ligated to the linearized pQE30 vector with the aid of T4 DNA ligase. The ligation mixture was introduced into *E. coli* XL1-blue cells by electroporation. Transformants were selected on Luria-Bertani agar supplemented with ampicillin to 150 μg/ml and tetracycline to 10 μg/ml. The structure of the resulting *asgA* expression plasmid (pYL2) was confirmed by restriction analysis of the plasmid DNAs and small-scale expression assays as described by the manufacturer of the QIAexpress system. To verify that the fusion was in frame, DNA sequencing with a SequiTherm cycle sequencing kit (Epicentre Technologies) was performed by using the primer 5′-GGAG CTGCGAAAGATGC-3′ to sequence across the cloning junction of pYL2.

Overproduction and purification of AsgA. Large-scale expression cultures were obtained as described in protocol 3 of the QIAexpress system with the modifications that tetracycline instead of kanamycin was added to Luria-Bertani broth, and the cultures continued to grow at 37°C for 3 h after induction by isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM final concentration). Overproduced recombinant AsgA protein was found to be located in cytoplasmic inclusion bodies. Therefore, the protein was purified under denaturing conditions with the aid of nickel-nitrilotriacetyl resin by following protocol 7 of the QIAexpress system with the modification of substituting 8 M urea for 6 M guanidine hydrochloride in buffer A (6 M guanidine hydrochloride, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8) and buffer F (6 M guanidine hydrochloride, 0.2 M acetic acid). Elution of the tagged protein was achieved by reducing the pH of the elution buffer from 5.9 to 2.0 in a step-wise fashion. We found that multiple polypeptides were

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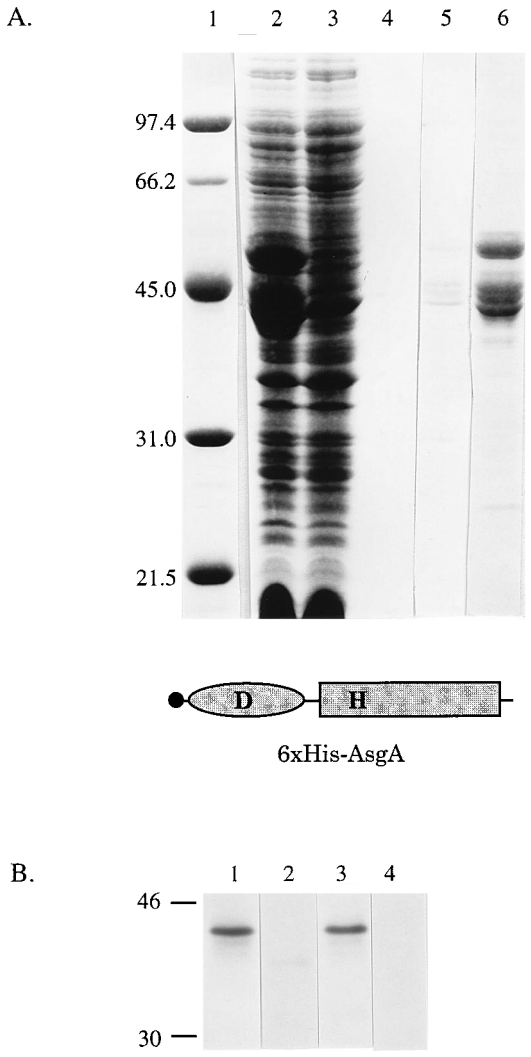

FIG. 1. Purification of AsgA protein. (A) AsgA was purified by Ni affinity chromatography as described in the text. Fractions were analyzed on an SDS–10% polyacrylamide gel and visualized with Coomassie blue stain (15). Lanes: 1, molecular mass markers (in kilodaltons); 2, cell extract of XL1-blue/pYL2 prepared after induction with IPTG; 3, cell extract of XL1-blue/pYL2 grown in the absence of IPTG; 4 to 6, proteins eluted during wash with buffer at pH 5.9 (lane 4), pH 4.5 (lane 5), and pH 2.0 (lane 6). The diagram below the gel illustrates the domain organization of the deduced AsgA amino acid sequence and follows the convention used by Parkinson and Kofoid (12). The oval represents the RR domain, and the rectangle represents the HPK domain (12). The black dot represents the 6xHis tag of recombinant AsgA. (B) Western immunoblot of AsgA in cell extracts from an *M. xanthus* wild-type strain and from a strain containing a kanamycin resistance gene inserted within *asg* (14). Growing and developing cells were collected and resuspended as described by Kroos et al. (7). Twelve microliters of each cell suspension was mixed with 3 μl of 5× sample buffer for SDS-polyacrylamide gel electrophoresis (15) and heated at 95°C for 7 min. Forty micrograms of protein from each cell preparation was subjected to SDS-polyacrylamide gel electrophoresis. AsgA was detected by using ECL Western blotting protocols (Amersham) and a 1:5,000 dilution of the immune serum and a 1:15,000 dilution of the secondary antibody (horseradish peroxidase-conjugated sheep anti-rabbit antiserum). Lanes: 1, extract from growing wild-type cells; 2, extract from growing cells containing the *asgA* disruption; 3, extract from developing (6-h) wild-type cells; 4, extract from developing (6-h) cells containing the *asgA* disruption. The 46- and 30-kDa molecular mass markers are indicated.

coeluted from the column (Fig. 1A, lane 6). All of these polypeptides appear to be induced following addition of IPTG (Fig. 1A, lane 2), which suggests that they are products of *asgA*. Furthermore, the polypeptides most likely contain the 6xHis tag and the intact N terminus of AsgA, because they bind to the nickel-nitrilotriacetyl resin. Two of the polypeptides migrate close together and exhibit apparent molecular masses of approximately 54.5 and 52.5 kDa. Three additional polypeptides migrate close together and have apparent molecular masses of 43 to 47 kDa. The predicted molecular mass of AsgA is 42,024 Da (14). It is known that the 6xHis tag may slow the migration of the recombinant protein on polyacrylamide gels and thus make it appear to be several kilodaltons larger than the equivalent untagged protein. There are at least two possible explanations for the multiple bands. First, the multiple polypeptides might be different phosphorylated forms of AsgA; second, the lower-molecular-mass polypeptides might be products of incomplete translation of *asgA* or degradative products of the full-length AsgA protein.

Polyclonal antibodies were raised against a fraction containing all five polypeptides. These antibodies recognize a 42-kDa protein that is present in extracts prepared from wild-type *M. xanthus* but is absent in extracts prepared from an *asgA* disruption strain (Fig. 1B). This result suggests that the purified protein is indeed AsgA and that the purification of multiple polypeptides is probably an artifact of the expression system.

To renature the recombinant protein, refolding was carried out by step-wise dilution of the urea by dialysis. Aliquots (5 to 10 ml) of eluted protein (0.5 to 1.0 mg/ml) were dialyzed for 10 h at 4°C with slow stirring against 500 ml of refolding buffer (30 mM Tris-HCl [pH 7.6], 200 mM KCl, 1 mM EDTA, 5 mM dithiothreitol, 10% [vol/vol] glycerol, 0.1% Triton X-100) (16) containing 6 M urea. The protein preparation was then dialyzed against 500 ml of refolding buffer containing 3 M urea. This process was carried out three more times, with a lower concentration of urea (2, 0.5, and 0 M) present in the refolding buffer each time. Dialysis continued for at least 6 h at each step. Finally, the protein preparation was dialyzed against refolding buffer without stirring overnight. Renatured proteins were split into 1-ml or 10-μl aliquots at 0.6 to 0.8 μg of protein per μl and stored at −90°C. The yield of protein from the purification was approximately 6% of the starting total protein. The percent renaturation of purified AsgA is unknown.

In vitro phosphorylation assays. Phosphorylation reactions were carried out in a 10-μl volume containing 0.66 μg of purified AsgA in phosphorylation buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES] [pH 8.0], 50 mM KCl, 0.5 mM EDTA, 2 mM dithiothreitol) supplemented with $MnCl_2$ to 1 mM. The reaction was initiated by adding a mixture of 1 to 2 μl of 10-μCi/μl [γ-^{32}P]ATP (6,000 Ci/mmol) (DuPont NEN) and 2.5 μl of 250 μM ATP, which gives an approximate final ATP concentration of 25 μM (40 to 80 Ci/mmol). After incubation for 5 to 10 min at room temperature, the reaction was stopped by addition of 4 μl of sodium dodecyl sulfate (SDS) loading buffer (0.32 M Tris-HCl [pH 6.8], 0.1 M EDTA, 10% [wt/vol] SDS, 40% [vol/vol] glycerol, 20% [vol/vol] β-mercaptoethanol, 0.02% [wt/vol] bromophenol blue). The samples were immediately heated for 5 min at 85°C and loaded onto an SDS–10% polyacrylamide gel.

Electrophoretically separated phosphoproteins were transferred from the polyacrylamide gel to a MagnaGraph nylon membrane (Micron Separations Inc.) or an Immobilon-P membrane (Millipore) by using a semidry electroblotter. After transfer, both the membrane and the SDS-polyacrylamide gel were briefly washed with water to eliminate nonspecifically bound label, dried, and then analyzed with a PhosphorImager. The membrane and the posttransfer gel were rendered for visualization by exposure to X-ray film for 1 to 4 days.

When purified AsgA was incubated with [γ-^{32}P]ATP at room temperature, it was labeled within minutes, suggesting

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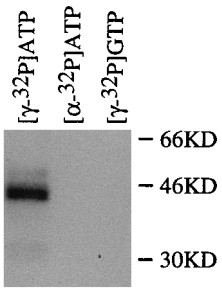

FIG. 2. Autophosphorylation of AsgA. Purified AsgA (0.66 μg) was incubated with [γ-^{32}P]ATP, [α-^{32}P]ATP, or [γ-^{32}P]GTP in the presence of 1 mM $MnCl_2$ under standard conditions for 10 min. Reaction products were subjected to SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane, and exposed to X-ray film with an intensifying screen at −90°C for 24 h.

that AsgA is an autokinase. (It should be noted that the purified protein preparation contains several different proteins that are probably different forms of AsgA. Therefore, it is not certain that the protein is an autokinase, because one species of protein in the mixture could catalyze phosphorylation of another.) This activity is heat labile; heating at 95°C for 5 min resulted in a nearly complete loss of activity (data not shown). No radioactive products were produced in the presence of either [α-^{32}P]ATP or [γ-^{32}P]GTP (Fig. 2), indicating that phosphorylation of AsgA is nucleotide specific and γ-phosphate specific. Interestingly, most of the label is found in the bands with apparent molecular masses of 43 to 47 kDa rather than in the bands with apparent molecular masses of 52.5 and 54.5 kDa. We know that the higher-molecular-mass polypeptides are present following the in vitro phosphorylation reaction because they can be visualized with Coomassie blue stain (data not shown).

Many HPKs require the presence of an appropriate concentration of certain divalent cations for their activities. For example, autophosphorylation of FrzE, an *M. xanthus* protein containing domains homologous to CheA and CheY, requires the presence of Mn^{2+} (10). To test whether divalent cations can stimulate the autokinase activity of AsgA, different cations were added to the phosphorylation reaction buffer and examined for their effects on AsgA activity. We found that AsgA was not detectably phosphorylated in the absence of divalent cations; however, when certain divalent cations were supplied at a concentration of 5 mM, AsgA was phosphorylated, with the degree of stimulation as follows: $Mn^{2+} > Mg^{2+} > Ca^{2+} > Fe^{2+}$ (data not shown). No phosphorylated AsgA was detected in the presence of Zn^{2+} or Cu^{2+}. All subsequent phosphorylation reactions were carried out in the presence of Mn^{2+}.

To further investigate the stimulatory effect of Mn^{2+}, the concentration of Mn^{2+} was varied from 0.1 to 200 mM and the levels of AsgA-phosphate under these conditions were determined (Fig. 3). A peak level of AsgA-phosphate is produced at 1 mM Mn^{2+}. The presence of either much lower or much higher concentrations of Mn^{2+} results in dramatically decreased production of AsgA-phosphate, implying that Mn^{2+} may act as a cofactor or activator of the enzyme. The range of Mn^{2+} concentrations required for AsgA activity is in the physiological-concentration range of Mn^{2+} in *E. coli* cells (4); therefore, the requirement of Mn^{2+} appears to be physiologically relevant.

A time course of AsgA phosphorylation was monitored to investigate the initial AsgA phosphorylation rate. As reported for several other sensor proteins (5, 16, 18), incorporation of γ-phosphate into AsgA occurs very rapidly, reaching about 70% of the maximum within 1.5 min (data not shown). Lowering the reaction temperature to 4°C did not detectably slow down the reaction; thus, we were unable to measure the initial kinetics of AsgA autophosphorylation.

Chemical stability of the phospholinkage. FrzE and ArcB are sensor proteins that, like AsgA, contain conserved HPK and RR domains (12). Chemical-stability assays have been performed to determine the class of the phosphorylated amino acid in both of these proteins following in vitro autophosphorylation reactions. Experiments with FrzE indicate that phosphorylated FrzE contains an aspartyl phosphate. It has been proposed that FrzE autophosphorylates on the conserved histidine residue of its HPK domain and transfers the phosphoryl group to the conserved aspartate of its RR domain (10). Similar chemical-stability experiments indicate that ArcB contains both a histidyl phosphate and an aspartyl phosphate. Iuchi and Lin (5) proposed a model for the signaling process by ArcB. Upon stimulation, ArcB undergoes autophosphorylation at the conserved histidine within the HPK domain. The phosphoryl group is transferred to the conserved aspartate in the receiver domain; this transfer is followed by another round of autophosphorylation at the conserved histidine. In this model, phosphorylation of the RR domain of ArcB is required for efficient transfer of the phosphoryl group from the histidine of ArcB to the aspartate of ArcA (5). We performed chemical-stability assays as described by McCleary and Zusman (10) to determine the class of amino acid residue that is phosphory-

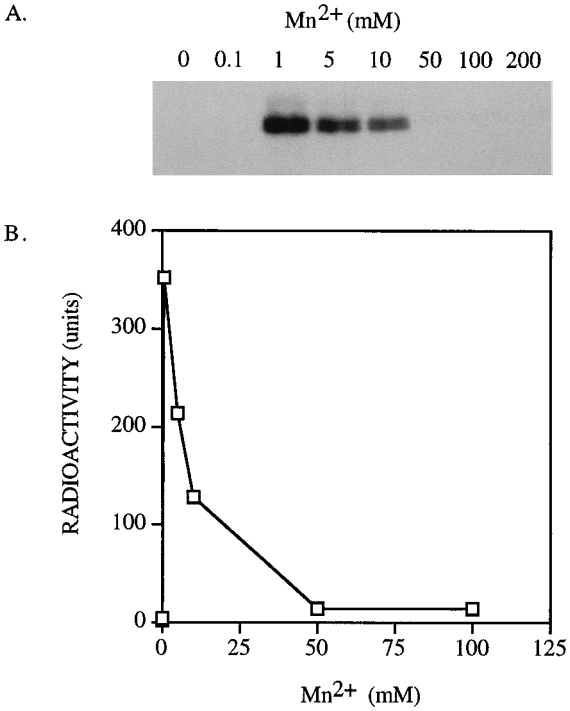

FIG. 3. Phosphorylation of AsgA at various concentrations of Mn^{2+}. Equal amounts of AsgA were incubated with [γ-^{32}P]ATP in the presence of the indicated concentration of $MnCl_2$ for 15 min and then subjected to SDS-polyacrylamide gel electrophoresis and transfer to an Immobilon-P membrane. (A) Autoradiograph of the phosphorylation blot after exposure to X-ray film with an intensifying screen for 3 days at −90°C. (B) Quantification of AsgA-phosphate produced at various concentrations of Mn^{2+}. The blot was exposed to a PhosphorImager plate for 42 h. The incorporation of γ-phosphate into AsgA was determined by using a Fujix Bio-Imaging analyzer BAS2000 (Fuji Photo Film Co.) and the MacBas V2.1 software program as described in the manufacturer's manual. The intensity of a radioactive protein is expressed in radioactivity units, which are values of photostimulated luminescence.

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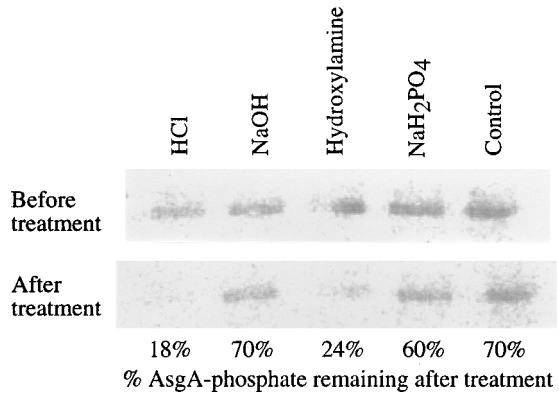

FIG. 4. Chemical stability of phosphorylated AsgA. Purified AsgA (0.66 μg) was phosphorylated under standard conditions for 10 min. Products from five identical reactions were loaded onto an SDS–10% polyacrylamide gel for electrophoresis and then transferred to an Immobilon-P membrane. The blot was placed on a PhosphorImager plate for 2 h for visualization and quantification of the radioactivity of AsgA-phosphate. Next, the blot was cut into five strips, each containing the radioactive products from one reaction. Each of four strips was incubated in one of four solutions (of 1 M HCl, 2 N NaOH, 0.8 M hydroxylamine [pH 7.0], or 50 mM NaH_2PO_4 [pH 7.0]) at 42°C for 2.5 h. One strip was left at room temperature without any treatment (control). After the 2.5 h of incubation, all strips were neutralized, briefly rinsed, air dried, and then exposed to the PhosphorImager plate for 2 h. Images of phosphoproteins before and after treatments are shown. Western immunoblotting assays with AsgA antibody were performed with all five strips to verify that protein was not stripped off the membranes during treatments (data not shown). The percentage of remaining radioactivity is shown.

lated in AsgA-phosphate formed in vitro. N-phosphorylated amino acids, including arginine, histidine, and lysine, are sensitive to hydrolysis in the presence of acid, hydroxylamine, or pyridine but are relatively stable to alkali. O-phosphorylated amino acids, including serine, threonine, and tyrosine, are all stable in strong acids. Acyl-phosphorylated amino acids, including glutamate and aspartate, are labile in both base and acid (2). The AsgA phospholinkage is stable in base but is acid and hydroxylamine labile (Fig. 4). These results indicate the presence of an N-linked phosphate such as histidyl phosphate; however, the phosphorylated amino acid has not been identified directly. Our results provide no evidence for the transfer of the phosphate from the conserved histidine in the HPK domain to the conserved aspartate in the RR domain in vitro. Further studies are required to determine the function of the AsgA RR domain and to identify putative signaling proteins that interact with AsgA in vivo.

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